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Son Maintains Accurate Splicing for a Subset of Human Pre-mRNAs

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Summary

Serine-arginine-rich (SR) proteins play a key role in alternative pre-mRNA splicing in eukaryotes. We recently showed that a large SR protein called Son has unique repeat motifs that are essential for maintaining the subnuclear organization of pre-mRNA processing factors in nuclear speckles. Motif analysis of Son highlights putative RNA interaction domains that suggest a direct role for Son in pre-mRNA splicing. Here, we used in situ approaches to show that Son localizes to a reporter minigene transcription site, and that RNAi-mediated Son depletion causes exon skipping on reporter transcripts at this transcription site. A genome-wide exon microarray analysis was performed to identify human transcription and splicing targets of Son. Our data show that Son-regulated splicing encompasses all known types of alternative splicing, the most common being alternative splicing of cassette exons. We confirmed that knockdown of Son leads to exon skipping in pre-mRNAs for chromatin-modifying enzymes, including ADA, HDAC6 and SetD8. This study reports a comprehensive view of human transcription and splicing targets for Son in fundamental cellular pathways such as integrin-mediated cell adhesion, cell cycle regulation, cholesterol biosynthesis, apoptosis and epigenetic regulation of gene expression.

Key words: Son, NREBP, pre-mRNA, Alternative splicing, SR proteins, Nuclear speckles

Introduction

Alternative splicing maximizes genome coding potential by generating diverse protein isoforms in eukaryotic cells. The antagonistic interplay among *trans*-acting RNA-processing factors modulates their binding to *cis*-acting elements in pre-mRNAs and controls the accuracy of splice-site selection. Defective pre-mRNA processing can lead to production of defective proteins if aberrant transcripts escape mRNA quality control (Fasken and Corbett, 2005; Fasken and Corbett, 2009), and many examples of disease have now been demonstrated as a result of improper splicing events (Ward and Cooper, 2010). Because these events can depend on individual splicing factors, it is crucial to understand what subsets of genes are regulated by individual splicing factors, and also to learn the functions of new splicing factors in constitutive and/or alternative splicing.

Among the best-characterized splicing factors are members of the SR protein family. SR proteins typically contain RNA recognition motifs and a serine-arginine (SR)-rich domain that mediate binding to RNA and other splicing factors during co-transcriptional splicing, although SR proteins also have many additional functions in other steps of gene regulation (reviewed by Shepard and Hertel, 2009; Zhong et al., 2010). The identity of classical SR proteins has been recently clarified, and nomenclature has been established based entirely on the sequence motif content of family members (Manley and Krainer, 2010). Proteomic analysis of nuclear bodies such as nuclear speckles identified new SR proteins whose motif content is highly suggestive of a role in pre-mRNA splicing (Mintz et al., 1999; Saitoh et al., 2004). One such protein, called Son, is important for maintaining proper organization of pre-mRNA processing factors in nuclear speckles (Sharma et al., 2010). An interesting feature of Son is that it contains multiple tandem repeat motifs that are unique to Son and could provide docking sites for simultaneous interaction with many partners. Two of these tandemly repeating units are serine rich, implicating phosphorylation in the regulation of Son activity.

The C-terminal domain of the largest subunit of RNA polymerase II is a well-documented example in which serine-rich tandem-heptad-repeat motifs provide scaffolding for co-transcriptional loading of RNA processing complexes onto pre-mRNAs (Misteli and Spector, 1999). The unique serine-rich tandem repeat motifs in the primary sequence, in addition to its putative RNA binding domains, led us to examine whether Son is present at transcription sites where it could have a role in co-transcriptional processing of pre-mRNAs. Alternative splicing of β-tropomyosin minigene (BTM) transcripts has been extensively studied, and exon 6 of the minigene is alternatively spliced in some cell types (Caceres et al., 1994; Guo et al., 1991; Helfman et al., 1988). Robust recruitment of splicing factors to BTM minigene transcripts was previously demonstrated in situ (Huang and Spector, 1996), and stable integration of this reporter minigene into HeLa cells generated a constitutively active

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transcription site (Prasanth et al., 2003), which we have now used as an alternative splicing reporter in situ.

Here we report that Son accumulates at a constitutively active β-tropomyosin minigene reporter transcription site along with other pre-mRNA splicing factors. By expressing YFP-tagged Son deletion mutants, we confirmed that localization of Son to this transcription site requires its N-terminus inclusive of the unique tandem repeats, whereas the RS domain, DSRBD and G-patch are all dispensable. Interestingly, alterations in nuclear speckle organization that occurred in Son-depleted cells did not affect the recruitment of other pre-mRNA splicing factors to this transcription site. We therefore used this system to study the effects of Son depletion on pre-mRNA transcription and splicing at this locus. Although Son depletion did not affect reporter transcript levels, it resulted in exon skipping in reporter transcripts. Because this result clearly pointed toward a role for Son in splicing, we carried out a genome-wide screen for human splicing targets of Son. We demonstrate that Son-depleted cells exhibit aberrant splicing of pre-mRNAs encoding the chromatin-modifying enzymes adenosine deaminase (ADA), histone deacetylase 6 (HDAC6) and histone lysine N-methyltransferase 8 (SetD8). This report identifies human transcripts in major metabolic and gene regulatory pathways that are modulated by Son, either at the transcript level and/or by alternative splicing. By demonstrating that Son is a splicing factor for many human pre-mRNAs, our study impacts a variety of disease-relevant pathways, and it opens new avenues for understanding how alternative splicing choices are regulated.

Results

The N-terminus of Son mediates its localization to a transcription site

Although Son is reported to be a nuclear speckle protein (Mintz et al., 1999; Wynn et al., 2000; Saitoh et al., 2004; Sharma et al., 2010), and it is present in the nuclear-insoluble fraction (Takata et al., 2009), it was not known whether Son is also recruited to transcription sites. To test this possibility, we used HeLa cells stably expressing a rat β-tropomyosin minigene (BTM HeLa cells; Fig. 1A) (Prasanth et al., 2003). The β-tropomyosin minigene (BTM) locus in these cells is typically detected as a single transcription site in each nucleus. RNA transcripts from the minigene were visualized by RNA fluorescence in situ hybridization (RNA-FISH) using a Texas-Red-conjugated probe targeting exon 5 (Fig. 1A). Dual-immunofluorescence labeling using antibodies against endogenous Son and essential splicing factor SRSF1/SF2/ASF showed that Son (Fig. 1B, panel C) and SRSF1/SF2/ASF (Fig. 1B, panel B) overlapped with reporter transcripts in situ (Fig. 1B, panel A). Similar results were observed with snRNP recruitment, because Son (Fig. 1C, panel K) and U1-70K (Fig. 1C, panel J) overlapped with reporter transcripts in situ (Fig. 1C, panel I). These data demonstrate that Son, SRSF1/SF2/ASF and U1-70K localize to the β-tropomyosin minigene transcription site. Importantly, proteins not previously shown to have functions in pre-mRNA transcription or splicing, including the rRNA processing factor fibrillarin (Fig. 1D) and PML protein (Fig. 1E), are not present at the transcription site.

To determine what is required for Son localization to the BTM transcription site, full-length and deletion mutants of Son (Sharma et al., 2010) (Fig. 2) were transfected into HeLa BTM cells. Forty-eight hours post transfection, the cells were seeded onto coverslips and labeled with Texas-Red-conjugated probes targeting exon 5 of the reporter transcripts. Antibodies targeting GFP were used to label YFP–Son mutants because YFP fluorescence did not survive the RNA-FISH procedure. YFP–Son colocalized with reporter transcripts at the transcription site (Fig. 2A–D). YFP–Son(1–2008), which contains the unique repeats and the RS domain but lacks putative RNA binding regions colocalized with BTM transcripts (Fig. 2E–H). YFP–Son(1–1493), which contains the unique repeats also colocalized with BTM transcripts (Fig. 2I–L). These data indicate that the smallest construct consisting primarily of a short N-terminal sequence and all of the tandem repeats mediate Son accumulation at the BTM transcription site, whereas the C-terminal domains, including the RS domain, DSRBD and G-patch are dispensable. Although these C-terminal domains are not essential for targeting Son to transcription sites, they might be important for the interaction of Son with other splicing factors or for recognition of RNA sequence elements in the pre-mRNA transcripts.

Son depletion alters splicing but not transcript levels of the β-tropomyosin minigene pre-mRNA

HeLa BTM cells were transfected with two independent siRNA duplexes previously shown to effectively reduce expression of Son (Son siGENOME1 or Son siGENOME4) (Sharma et al., 2010), or with mock (vehicle) or control (luciferase) siRNAs. Forty-eight hours post transfection, RNA was extracted from these cells and qRT-PCR was performed to confirm Son depletion. The effects of Son depletion on transcript levels and spliced isoforms of BTM reporter RNA was assessed using various primer sets targeting different exon–exon and exon–intron regions of the minigene transcripts as shown in Fig. 3A. The total level of BTM transcripts was not significantly different between Son-depleted and control cells when measured during the quantitative log phase using qRT-PCR (Fig. 3B, primer sets A–D). These measurements show that there were similar total amounts of BTM transcripts in the total RNA samples; however, this does not reflect the relative amounts of BTM splice variants in each RNA sample. To determine which splice variants were present, final qRT-PCR products amplified using primer set A (targeting exon 5 through exon 7 of the BTM reporter transcripts) were applied to polyacrylamide gels and showed several splice products (Fig. 3C). In mock and control conditions, we observed PCR products corresponding to unspliced BTM transcripts, fully spliced BTM transcripts and BTM transcripts in which exon 6 was skipped. Interestingly, Son-depleted cells showed a major shift from inclusion of exon 6 to skipping of exon 6 on the BTM transcripts, suggesting that Son is involved in regulating alternative splicing.

The altered splicing pattern we observed would be most simply explained if synthesis of reporter pre-mRNAs became uncoupled from splicing following Son depletion, because this might alter the extent of splicing factor association with transcripts and this would affect splice site selection. We performed dual RNA-FISH followed by immunofluorescence localization of Son to determine whether BTM transcripts lacking exon 6 are present at the BTM transcription site in Son-depleted cells. Hybridization of a Texas-Red-conjugated oligonucleotide probe complementary to exon 5 indicated robust transcription at the BTM locus in all nuclei for all samples (Fig. 4B,G,L), which was consistent with qRT-PCR results in Fig. 3B. Immunofluorescence labeling confirmed that Son was significantly reduced in cells treated with siRNAs to knock down Son (Fig. 4F,K) as compared with cells treated with a control oligo (Fig. 4A). A Cy5-labeled oligonucleotide probe complementary to the splice junction of exons 5 and 7 (exon

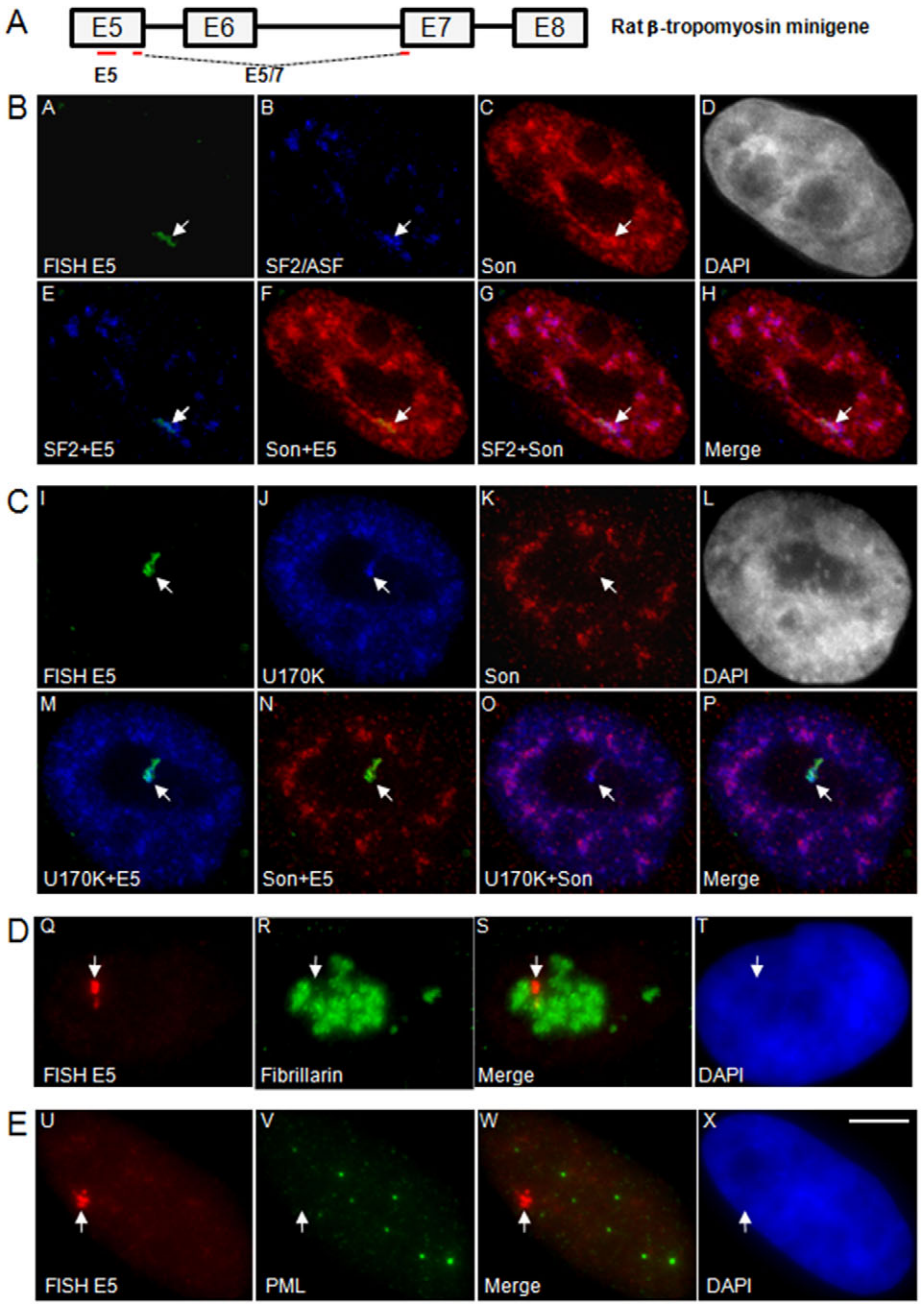

Fig. 1. Son colocalizes with splicing factors at the BTM transcription site. (**A**) Structure of rat β-tropomyosin minigene. (**B**,**C**) BTM HeLa cells stably transfected with the rat β-tropomyosin minigene. BTM HeLa cells were processed for RNA-FISH localization (panels B and C) of reporter RNA (panels A and I) and immunofluorescence localization of Son (panels C and K) and either splicing factor SRSF1/SF2/ASF (B) or U1-70K (J). 50/50 cells scored in each experiment showed Son localization at the BTM transcription site. (**D**) Fibrillarin is not localized at the BTM transcription site. (**E**) PML protein is not localized at the BTM transcription site. Arrows indicate the BTM transcription site. DNA is stained with DAPI. Scale bar: 5 μm.

junction probe) was used to detect transcripts in which exon 6 is skipped. This exon junction probe was not detectable at transcription sites in control cells (Fig. 4C); however, it was clearly detectable at the BTM transcription site in Son-depleted cells (Fig. 4H,M). Furthermore, the exon junction probe colocalized with the BTM transcripts labeled with the exon 5 probe (Fig. 4I,N). Taken together, the results in Figs 3 and 4 demonstrate that BTM transcripts in Son-depleted cells are at the transcription site when exon 6 is skipped, and that Son is required for proper splicing of BTM reporter minigene transcripts.

Splicing factors are recruited to the β-tropomyosin minigene locus in Son-depleted cells

Nuclear speckles are storage and assembly sites for splicing factors, and their integrity is essential for coupling transcription and pre-mRNA splicing (Sacco-Bubulya and Spector, 2002; Spector and Lamond, 2011). Our earlier studies showed that depletion of Son alters the organization of pre-RNA processing factors (U1-70K, SRSF1/SF2/ASF, SC35, Magoh), polyadenylated RNA and lncRNA (MALAT1) found in nuclear speckles (Sharma et al., 2010; Tripathi et al., 2010). A change in splice site selection would also be simply explained if splicing factors could no longer reach transcription sites following Son knockdown. To determine whether changes in nuclear speckle organization observed in Son-depleted cells disrupted recruitment of splicing factors to the BTM transcription site, we performed RNA-FISH in Son-depleted cells using BTM exon 5 probes followed by dual immunofluorescence with antibodies against Son and U1-70K (Fig. 5) or Son and SRSF1/SF2/ASF (Fig. 6). Son, SRSF1/SF2/ASF and U1-70K were all recruited to the BTM transcription site when cells were

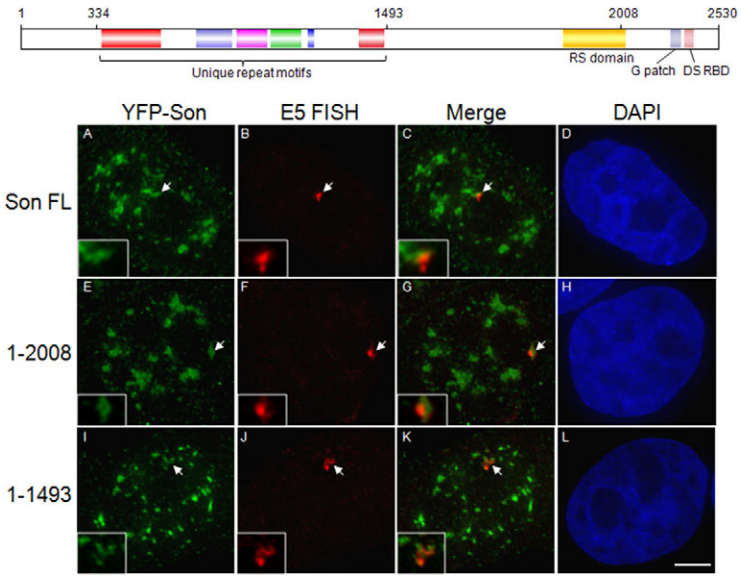

Fig. 2. Son repeats mediate localization of Son at the BTM locus. Top panel indicates boundaries of deletion in full-length Son. BTM HeLa cells were transiently transfected with YFP–Son full-length (1–2530) (**A**–**D**), or deletion constructs truncated after domain boundaries as indicated in the top panel: YFP–Son(1–2008) (**E**–**H**), YFP–Son(1–1493) (**I**–**L**). The cells were then processed for RNA-FISH localization of reporter RNA (B,F,J) and immunostaining of YFP. Following FISH, the cells were fixed again and processed for immunofluorescence for immunolocalization of YFP (A,E,I). Arrows indicate the BTM transcription site. 50/50 cells scored for each construct showed localization at the BTM transcription site. DNA is stained with DAPI. Scale bar: 5 μm.

transfected with control siRNAs (Fig. 5C, Fig. 6C). Son was significantly reduced in cells treated with Son siRNAs (Fig. 5F,K, Fig. 6F,K) compared with cells treated with control siRNA (Fig. 5A, Fig. 6A). Although U1-70K (Fig. 5H,M) and SRSF1/SF2/ASF (Fig. 6H,M) showed the expected change in their subnuclear localization following depletion of Son (note the appearance of nuclear speckles as a torroid phenotype indicated by arrowheads) (Sharma et al., 2010), both splicing factors were localized to the BTM transcription site (Fig. 5I,N, Fig. 6I,N). These results show that reorganization of nuclear speckles in Son-depleted cells does not prevent splicing factors from accumulating at the BTM transcription site.

Son depletion alters the abundance of a subset of human mRNAs

Proteomic analysis of nuclear speckles showed that the majority of nuclear speckle proteins are involved in pre-mRNA processing or RNA polymerase II transcription. Previous reports showed that Son represses transcription of hepatitis B virus (HBV) by binding to a negative regulatory element in the HBV core promoter (Sun et al., 2001), and Son is also required for proper influenza virus trafficking and RNA synthesis (Karlas et al., 2010). Gene repression by Son was also recently reported in mice, because Son interacts with a negative regulatory element in the gene promoter for growth hormone secretagogue receptor, which is involved in glucose metabolism regulation (Komori et al., 2010). Son-depleted cells did not show significantly different Br-UTP labeling of nascent transcripts compared with control cells (Sharma et al., 2010). But if only a small subset of genes is regulated by Son, or if equal numbers of gene transcripts are upregulated or downregulated in Son-depleted cells, then global labeling of nascent transcripts by Br-UTP incorporation might not detect these changes. With evidence that Son represses transcription of viral and mouse mRNAs, we wanted to identify Son-dependent changes in transcription and splicing genome wide. To that end, we performed five experimental replicates and compared transcript profiles of HeLa cells transfected with either siRNA against Son or a control siRNA. Five array repetitions were performed using Affymetrix Exon Array 1.0 and RNA isolated from cells treated for 48 hours with Son siRNA duplexes or control siRNA duplexes; longer treatment with siRNA duplexes was previously shown to cause growth arrest (Sharma et al., 2010; Huen et al., 2010) and significant increase in cell death (Huen et al., 2010). Son was significantly knocked down at 48 hours compared with the control samples in all five sets of RNA samples used for the using exon arrays (Fig. 7A).

The change in transcript level for specific genes was subtle, which is consistent with our published results that global transcription is not significantly altered in the absence of Son (Sharma et al., 2010). Exon array results suggest that depletion of Son caused a change in levels of transcript of genes involved in various biochemical processes, as indicated in Fig. 7B. Tables 1 and 2 list genes showing decreased or increased transcript levels. Our data indicate that Son might participate not only in gene repression as previously reported (Karlas et al., 2010; Komori et al., 2010), but also in gene activation, because Son depletion caused a decrease in the expression of several genes. In total, 568 targets showed at least a twofold decrease of expression after Son depletion compared with control cells, whereas 359 targets showed at least a twofold increase. The complete list of target genes is available at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/).

We validated several exon array hits by qRT-PCR, for both upregulated and downregulated mRNAs. Validations were performed on total RNA isolated from cells treated with control siRNAs or two independent siRNAs targeting Son (siGENOME1 and siGENOME4), and for this set of experiments, qRT-PCR primers were targeted within exons whose inclusion was unaffected by depletion of Son. Upregulated mRNAs included *GEMIN5*, interleukin 1A (*IL1A*), tumor necrosis factor receptor superfamily member 21 (death receptor 6; *TNFRSF21*), cyclin G1 (*CCNG1*), cytochrome p450 (*CYP1B1*), all of which are upregulated 2- to 2.5-fold in Son-depleted cells. Downregulated mRNAs include the cardiac muscle troponin (*TNCC1*) mRNA which is reduced by approximately 60% after Son depletion compared with levels in control samples, and cyclin-dependent kinase 5 (*CDK5*), which was reduced nearly 50% compared with control samples (Fig. 7C). The data in Fig. 7 clearly show that cells treated separately with two independent siRNA duplexes targeted against Son exhibit similar changes in mRNA levels for these transcripts, and are consistent

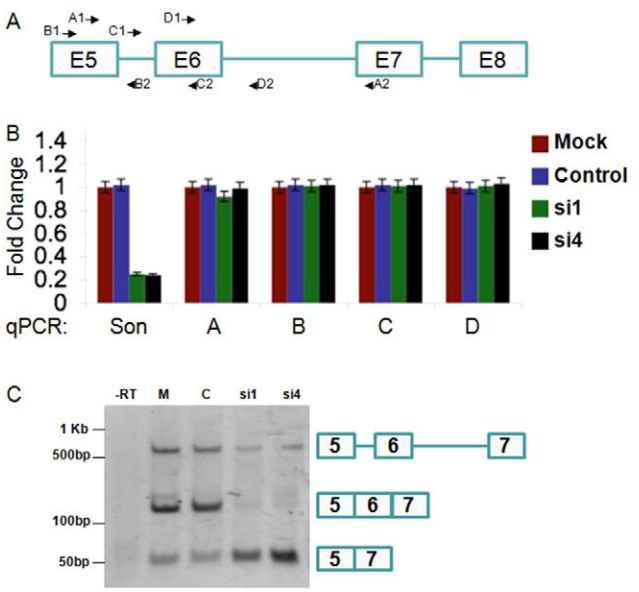

Fig. 3. Son depletion alters splicing but not mRNA levels of BTM reporter transcripts. (**A**) Schematic representation of the rat β-tropomyosin minigene in BTM HeLa cells that includes genomic sequence from exon 5 through exon 8. Introns are represented as thin lines connecting the exons. qPCR primers sets targeting BTM transcripts are represented by letters as follows: A1 and A2 span exon 5 to exon 7; B1 and B2 span exon 5 to intron 6; C1 and C2 span intron 5 to exon 6; and D1 and D2 span exon 6 to intron 6. *SON* mRNA levels were detected by qPCR primers specific to *SON* mRNA. (**B**) BTM HeLa cells were mock transfected with transfection vehicle only (Mock), or 60 pmoles of either control siRNA (C), *SON* siRNA1 (si1), or *SON* siRNA4 (si4). The graph shows total qRT-PCR product amplified using various primer pairs indicated on the *x*-axis (three technical replicates per bar), and is representative of three separate experiments. (**C**) qRT-PCR products from reactions using primer set A (exon 5 to exon 7) were analyzed using native polyacrylamide gel electrophoresis to examine alternative spliced products; corresponding products are shown on the right. –RT indicates reactions in which reverse transcriptase was omitted.

with changes on our exon array, thereby validating our gene expression analysis of Son-depleted cells.

Son maintains proper mitotic spindle organization at metaphase

Depletion of Son was shown to cause metaphase arrest in HeLa cells in our studies (Sharma et al., 2010) as well as in subsequent studies (Huen et al., 2010; Ahn et al., 2011). This increase in the number of mitotic cells is easily detected by microscopy, as shown in Fig. 8A. Son depletion was also shown to activate the MAD2 spindle assembly checkpoint and cause defects in chromosome alignment (Huen et al., 2010). Additionally, a genome-wide screen by Nousiainen and colleagues (Nousiainen et al., 2006) indicated that Son is a phosphoprotein of the mitotic spindle apparatus, suggesting that post-translational modification of Son could have important implications for mitotic cells; however, specific mitotic spindle defects have not yet been reported. Our microarray analysis showed changes in transcripts for many cell cycle regulators [including Katanin p80 subunit B1 (*KATNB1*), microtubule-associated protein 2 (*MAP2*), histone deacetylase 6 (*HDAC6*) and cyclin-dependent kinases such as *CDK6* and *CDK2*]. A similar but not completely overlapping set of mitotic regulator transcripts targeted by Son was recently reported by Ahn and co-workers (Ahn et al., 2011). Although the same trends in transcript upregulation or downregulation were seen for specific transcripts in both data sets, there were also differences in several specific transcripts identified by the two separate studies. Whereas Ahn and colleagues (Ahn et al., 2011) identified constitutive splicing problems in several cell cycle regulator transcripts, our exon array data shows additional information regarding alternative splicing regulation for some of these gene targets (see Discussion).

Because it is clear that HeLa cells show metaphase arrest following depletion of Son, we looked for structural changes in cellular organization through the cell cycle that would be consistent with the altered expression of cell cycle regulator

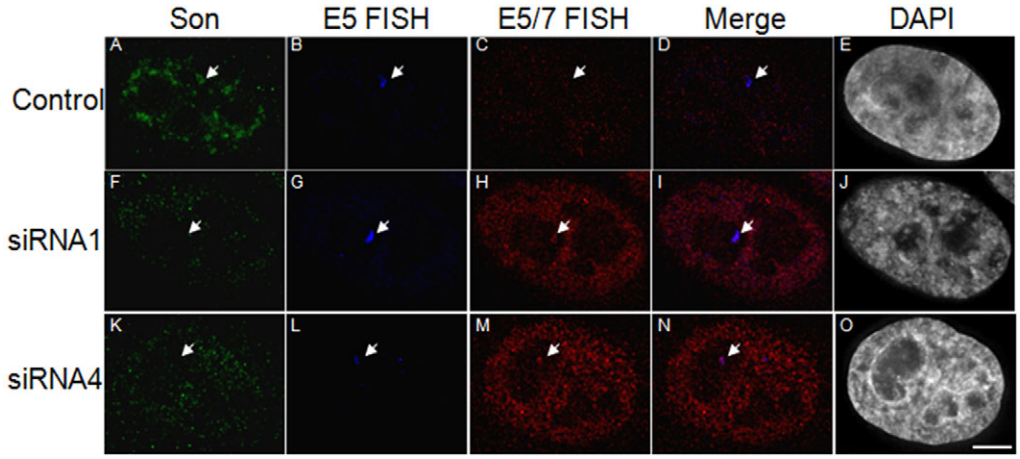

Fig. 4. Exon 6 skipping on BTM transcripts is detected only after Son depletion. (**A–O**) BTM HeLa cells were transfected with mock (M) transfection (vehicle), or 60 pmoles of either control siRNA (C), *SON* siRNA1 (si1) or *SON* siRNA4 (si4). 48 hours after transfection, the cells were processed for dual RNA-FISH localization of the BTM transcription site (Texas-Red-conjugated exon 5 probe; panels B, G and L) and transcripts in which exon 6 is skipped (Cy5-conjugated exon 5/7 probe; panels C, H and M) followed by immunofluorescence localization of Son (panels A, F and K). For controls, 50/50 cells showed strong E5 but weak E5/7 labeling, whereas 23/25 si1-treated and 25/25 si4-treated cells showed strong hybridization for both probes. Arrows indicate the BTM transcription site. Scale bar: 5 μm.

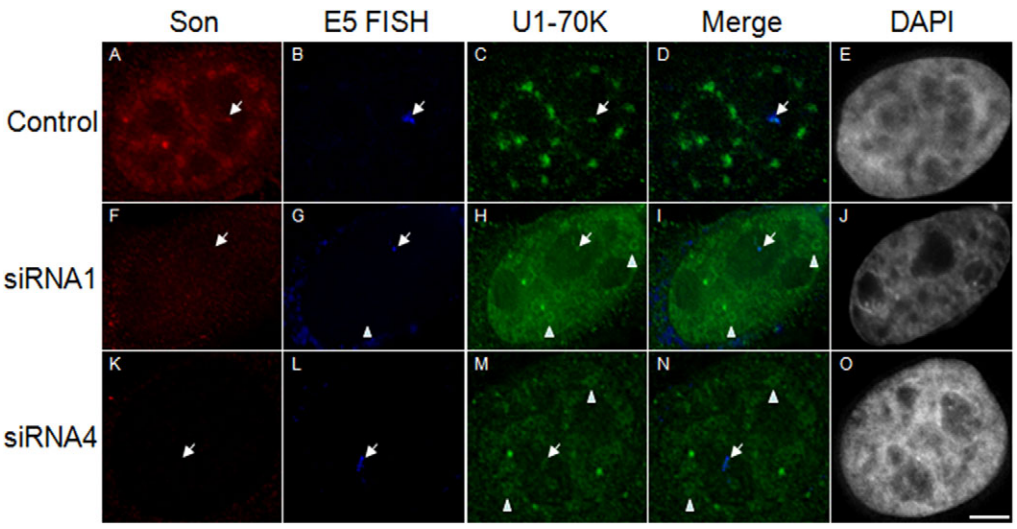

Fig. 5. Son depletion does not prevent recruitment of U1-70K to the BTM transcription site. (**A–O**) BTM HeLa cells were transfected with vehicle (M) or 60 pmoles of either control siRNA (C), *SON* siRNA1 (F–J) or *SON* siRNA4 (K–O). At 48 hours post transfection, the cells were processed for RNA-FISH localization of reporter RNA (B,G,L) followed by immunofluorescence localization of Son (A,F,K) and splicing factor U1-70K (C,H,M). Arrows indicate the BTM transcription site. DNA was stained with DAPI. Scale bar: 5 µm.

transcripts. In our study, the most obvious phenotypic difference that set Son-depleted cells apart from controls was the disorganized appearance of microtubules in the mitotic spindle apparatus (Fig. 8). Although control cells showed a compact mitotic spindle (Fig. 8D,E), prometaphase and metaphase cells treated separately with two independent siRNAs to knock down Son showed a disarrayed mitotic spindle apparatus, with many microtubules falling outside of the normal spindle boundaries (Fig. 8B). At metaphase, spindle microtubules were disorganized (arrows in Fig. 8F) and often showed a wavy appearance (arrows in Fig. 8H,I). Interpolar distance measurements of the spindle showed that average interpolar distance was 2–3 µm longer in Son-depleted metaphase cells (with average distance measuring 13.5 µm and 12.8 µm in Son-depleted cells versus 10.8 µm in control cells). Although the spindle organization was significantly altered following Son depletion, interkinetochore distance measurements were similar in all conditions (K.T.-M. and P.A.B., unpublished results), which indicates that spindle tension is not significantly altered despite the disorganized appearance of microtubules. Taken together, these results demonstrate that Son is important for proper organization of the spindle apparatus in human cells during metaphase, which probably occurs by indirect mechanisms given the alterations in transcripts encoding cell cycle regulators observed in Son-depleted cells that we and others have detected (see Tables 1–3 and Discussion) (Ahn et al., 2011).

Son depletion alters splicing of endogenous pre-mRNAs

In addition to the changes in gene expression observed upon Son depletion, we also observed changes in 1061 genes showing exon inclusion or exclusion, and a total of 2067 splicing events. Genes that demonstrated the most significant changes in splice site selection based on our analysis are listed in Table 3. Upon comparison of genes with changes in expression with those that exhibited changes in splice site selection due to alternative splicing, it was evident that they do not correlate. We detected splicing defects in 143 of 359 genes for which transcripts showed at least twofold upregulation (40%), we detected 61 splicing defects in 568 genes for which transcripts decreased at least twofold (11%) and we detected splicing defects in 857 genes that showed no changes in transcript level. With the exception of *GEMIN5* mRNA, levels of splicing factor transcripts (such as SR proteins and snRNP proteins) were not changed, which is consistent with our previous observations that depletion of Son does not alter the abundance of splicing factors SRSF1/SF2/ASF or U1-70K (Sharma et al., 2010).

Based on our array data, genes that exhibit changes in alternative splicing upon Son depletion include genes involved in

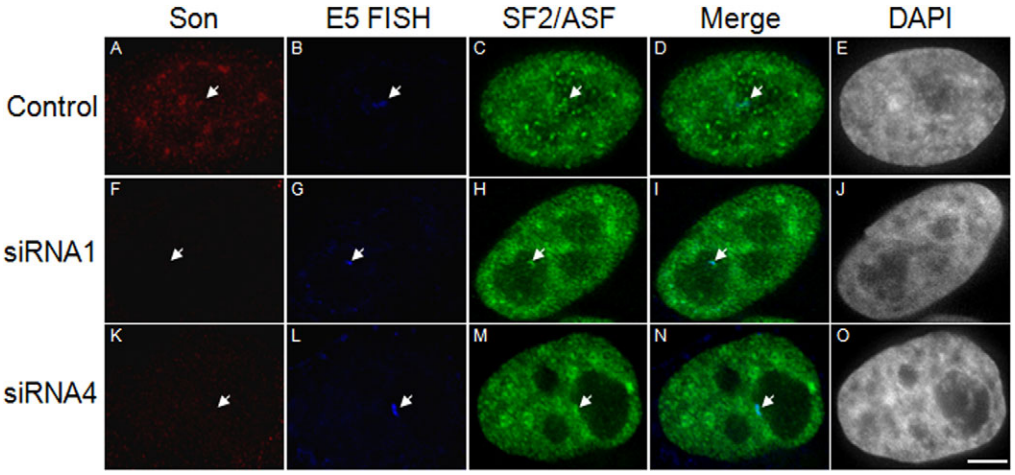

Fig. 6. Son depletion does not prevent recruitment of SRSF1/SF2/ASF to the BTM transcription site. (**A–O**) BTM HeLa cells were transfected with mock (M) transfection (vehicle), or 60 pmoles of either control siRNA (C), *SON* siRNA1 (F–J) or *SON* siRNA4 (K–O). 48 hours post transfection, the cells were processed for RNA-FISH localization of reporter RNA (B,G,L) followed by immunofluorescence localization of Son (A,F,K) and splicing factor SRSF1/SF2/ASF (C,H,M). Arrows indicate the BTM transcription site. DNA is stained with DAPI. Scale bar: 5 µm.

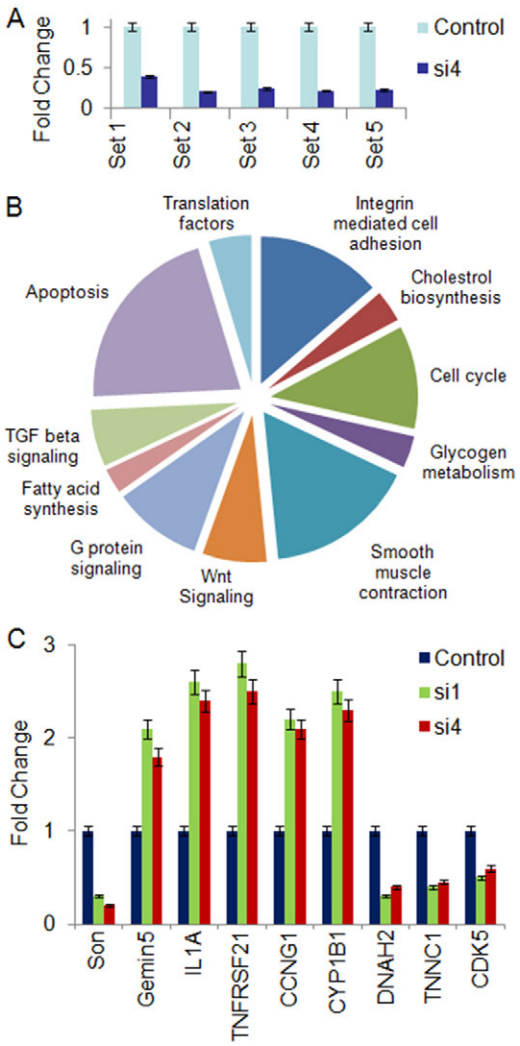

Fig. 7. Validation of several genes that show a change in transcript level after Son depletion in HeLa cells. (**A**) Five replicate total RNA samples isolated for exon array analysis indicate that Son is significantly reduced in HeLa cells treated with *SON* siRNA4 (si4) compared with control (C). GAPDH primers were used as an internal control. (**B**) Genes affected by altered mRNA levels or splicing following Son depletion categorized according to the cellular pathway affected. (**C**) qRT-PCR was performed in triplicate to validate mRNAs that are upregulated (*GEMIN5*, *IL1A*, *TNFRSF21*, *CCNG1* and *CYP1B1*) or downregulated (*DNAH2*, *TNNC1* and *CDK5*) after Son depletion with two separate siRNAs (si1 and si4) targeting *SON* compared with control siRNA (C). Results are means ± s.d.

chromatin modifications. These were of special interest because Son was recently identified as a pluripotency factor in human embryonic stem cells (Chia et al., 2010). Because maintenance of pluripotency is affected by epigenetic reprogramming, we were especially interested to know whether Son controls splicing for epigenetic regulators. Therefore, we chose to validate incorrect splicing for adenosine deaminase (*ADA*), *HDAC6* and histone lysine N-methyltransferase 8 (*SETD8*). Quantitative RT-PCR was used to detect various transcripts using primer pairs indicated in Fig. 9. Amplification spanned exons 8–10 of *ADA* transcripts (Fig. 9A), exons 26–29 of *HDAC6* transcripts (Fig. 9B), and exons 1–3 of *SETD8* transcripts (Fig. 9C). In agreement with our microarray analysis [and data from Ahn and colleagues (Ahn et al., 2011)], there was no significant difference observed in total transcript levels between the Son-depleted RNA samples and controls (Fig. 9D). However, Son-depleted samples showed a major shift in product toward exon skipping in these transcripts based on the size of PCR products. For *ADA* transcripts, Son-depleted samples show PCR products of the expected size for skipping of exon 9 (Fig. 9A). For *HDAC6*, Son-depleted samples show products of sizes consistent with skipping of exons 27 and 28 (Fig. 9B). In the case of *SETD8*, products were of the size expected for skipping of exon 2 (Fig. 9C). Exon skipping was reproducible in all cases using two separate siRNAs targeting Son, minimizing the possibility that altered splicing of these transcripts was an off-target effect. From these results, we conclude that Son maintains proper splice site selection in transcripts encoding these three important chromatin-modifying enzymes.

Discussion

Son has unique sequence features

The nuclear speckle localization of Son, its motif content and its localization along with other splicing factors to our constitutively active BTM transcription site are consistent with Son having pre-mRNA processing functions. A role for Son in pre-mRNA splicing has been suggested previously by the presence of an RS domain and putative RNA binding domains (Saitoh et al., 2004; Sharma et al., 2010), its cellular localization in nuclear speckles and mitotic interchromatin granule clusters (Wynn et al., 2000; Saitoh et al., 2004; Huen et al., 2010) as well as its interaction with a known splicing factor, SRrp53 (Cazalla et al., 2005). Here we show that Son is present at a transcription site where it maintains proper splicing of reporter transcripts. In addition to several putative RNA-binding motifs, tandem repeat motifs unique to Son are important for subnuclear organization of splicing factors (Sharma et al., 2010) and are required for targeting Son to transcription sites (this report).

Appropriate expression of protein coding genes in mammalian cells requires complex nuclear choreography. Pre-mRNA processing complexes are recruited to transcription sites for capping, splicing and polyadenylation, yielding mature mRNA transcripts for nuclear export (Bentley, 2005; Hirose and Manley, 2000; Lewis and Tollervey, 2000). Pre-mRNA processing in human nuclei is orchestrated by phosphorylation of the C-terminal domain of the largest RNA polymerase II (RNAPII) subunit (Bentley, 2005; Egloff and Murphy, 2008; Hirose and Manley, 2000; Lewis and Tollervey, 2000; Misteli and Spector, 1999; Phatnani and Greenleaf, 2004; Rosonina and Blencowe, 2004), which controls the initiation, elongation and termination or transcription (Brookes and Pombo, 2009; Meinhart et al., 2005; Phatnani and Greenleaf, 2006) and also promotes co-transcriptional loading of RNA processing factors onto nascent transcripts (reviewed in Hirose and Manley, 2000; Lewis and Tollervey, 2000; Bentley, 2005; Misteli and Spector, 1999). Two of the unique repeat motifs in Son have the consensus sequences MDSQMLASST and SMMSSAYERS. Although there is no direct sequence similarity between the repeats in Son and the 52 YSPTSPS repeats in the RNAPII CTD, the tandemly arrayed repeats in Son might serve a similar role to the RNAPII CTD heptad repeats in co-transcriptional pre-mRNA processing. First, the serine-rich and arginine-rich tandem repeats suggest that Son might be regulated by phosphorylation or methylation, respectively; the latter was recently shown for RNAPII CTD

Table 1. Genes showing increase in transcript levels after depletion of Son

Symbol	Name	Fold change	*P* value
AKR1B10	Aldo-keto reductase family 1 member B10	10.00	1.22E-05
PRG4	Proteoglycan 4 precursor (Lubricin)	9.59	1.36E-07
AC097065.2-2	Novel ScRNA pseudogene	8.43	1.19E-05
VTCN1	V-set domain-containing T-cell activation inhibitor 1 precursor	7.66	1.09E-05
CXorf52	Uncharacterized protein CXorf52	5.88	8.90E-05
AC132872.17-2	Putative uncharacterized protein ENSP00000380631 fragment	5.49	6.35E-07
AKR1C3	Aldo-keto reductase family 1 member C3	5.33	1.64E-04
AKR1C1	Aldo-keto reductase family 1 member C1	5.29	3.59E-07
OPN3	Opsin-3	4.86	4.16E-06
TNFRSF21	TNFR-related death receptor 6	4.39	1.75E-06
IL1A	Interleukin-1 alpha precursor	4.30	1.39E-03
CYP1B1	Cytochrome P450 1B1	3.46	4.46E-06
CCNG1	Cyclin G1	3.36	1.07E-03
GEMIN5	Gem-associated protein 5	3.23	1.25E-05

(Sims et al., 2011). In addition, the requirement of Son for maintaining proper splicing could involve interaction with splicing factors, where it could be a modular landing pad for binding subsets of splicing factors at transcription sites.

Son affects alternative splicing choices

SR proteins are essential for splice site selection as part of the exon definition complex of the spliceosome. They bind to exon splicing enhancers or intron splicing enhancers and promote assembly of the spliceosome machinery at nearby splice sites. SR proteins are antagonized by hnRNPs that bind to exonic splicing silencers and intronic splicing silencers (Black, 2003; Jurica and Moore, 2003; Liu et al., 1998; Mayeda et al., 1999; Wang et al., 2006; Zuo and Maniatis, 1996). Most SR proteins are recruited to transcription sites following phosphorylation of serines in their RS domain (Bentley, 2005; Egloff and Murphy, 2008; Hirose and Manley, 2000; Lewis and Tollervey, 2000; Misteli and Spector, 1999; Phatnani and Greenleaf, 2004; Rosonina and Blencowe, 2004). Although the RNA recognition motifs (RRMs) of the SR proteins help them to interact with the ESE and ISE regions of the nascent RNA, the RS domain is involved in recruiting the snRNPs to the splice site by protein–protein interactions (Black, 2003; Jurica and Moore, 2003; Liu et al., 1998; Mayeda et al., 1999; Wang et al., 2006; Zuo and Maniatis, 1996). We hypothesize that the repeats of Son could simultaneously bind multiple factors to enhance their potential interactions, or it might sequester factors to modulate their local concentrations at transcription sites. It is well known that splicing regulation is affected by modulation of SR protein levels; however, depletion of Son did not affect recruitment of splicing factors to the BTM locus. So splicing factors might not be dependent on Son to bring them from speckles to reach transcripts. Rather, the presence of Son at transcription sites might help to control the availability of splicing factors to participate in splicing by sequestration versus release within the vicinity of the transcription site. This idea would be consistent with the model that splicing factor subcomplexes are subsequently assembled into a variety of complexes as splicing progresses, and that these pathways and splicing outcomes might be reversed or altered if influenced by local subnuclear environment (Rino and Carmo-Fonseca, 2009).

Knockdown of Son caused a change in splicing of not only a constitutively expressed rat β-tropomyosin minigene reporter, but also of many endogenous genes. However, the speckle reorganization of pre-mRNA processing factors such as U170K and SRSF1/SF2/ASF after Son depletion does not affect their localization to the BTM transcription site, which is similar to other studies where knockdown of the nuclear-speckle-localized 7SK RNA changed speckle structure but did not affect recruitment of splicing factors to a transcription site (Prasanth et al., 2010). Son depletion also does not affect the protein expression level of these proteins (Sharma et al., 2010) and our array data suggests that splicing factor transcripts are generally unaltered by Son knockdown. Because exon 6 skipping on BTM transcripts cannot simply be attributed to an absence of SRSF1/SF2/ASF, it is more likely that proper splicing of exon 6 requires direct participation of Son at the transcription site. This data also strengthens the idea that BTM exon 6 skipping is a direct result of Son depletion and not an off-target effect caused by

Table 2. Genes showing decrease in transcript levels after depletion of Son

Symbol	Name	Fold change	*P* value
STXBP2	Syntaxin-binding protein 2	–7.05	5.80E-07
GNG11	Guanine nucleotide-binding protein gamma-11 precursor	–6.03	2.03E-04
SLC12A3	Solute carrier family 12 member 3	–6.18	1.59E-06
ST6GAL1	β-Galactoside α-2,6-sialytransferase 1	–5.86	2.61E-05
TPM4	Tropomyosin α-4 chain	–5.72	3.54E-05
WDR54	WD repeat-containing protein 54	–5.61	1.51E-06
PLAT	Tissue-type plasminogen activator precursor	–5.41	2.70E-07
SNCA	α-Synuclein	–5.20	3.00E-07
TINAGL1	Tubulointerstitial nephritis antigen-like precursor	–5.11	5.08E-06
QARS	Glutaminyl-tRNA synthetase	–4.86	1.65E-08
CDK5	Cell division protein kinase 5	–4.16	5.71E-07
TNNC1	Troponin C, slow skeletal and cardiac muscles	–4.05	4.84E-06
DNAH2	Dynein heavy chain2, axonemal	–3.32	5.56E-05

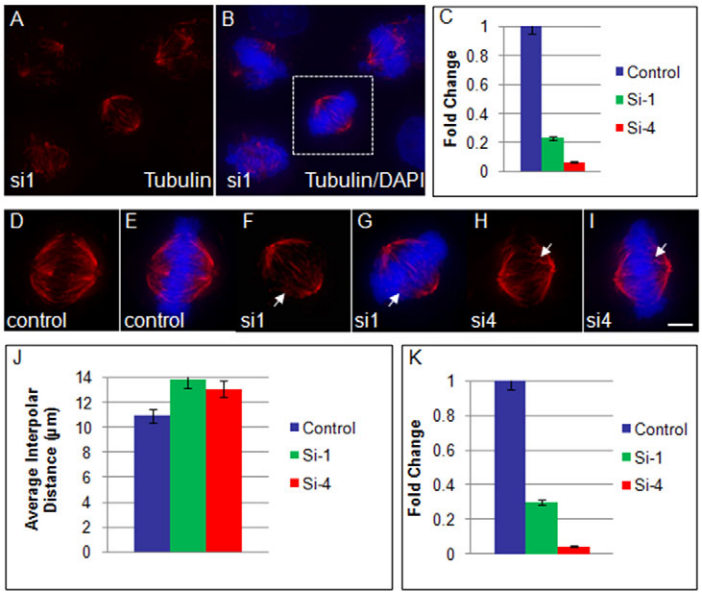

Fig. 8. Knockdown of Son leads to mitotic spindle defects. HeLa cells were transfected with 60 pmoles of either control siRNA (C), *SON* siRNA1 (si1) or *SON* siRNA4 (si4). 48 hours post transfection, the cells were processed for immunolabeling of α-tubulin. (**A**,**B**) Son-depleted samples show a marked increase in the number of prometaphase–metaphase cells and reduced *SON* mRNA (**C**). Microtubules seen in the control mitotic spindle (**D**,**E**) are disarrayed in Son-depleted metaphase cells (**F**–**I**). (**J**) Average interpolar distance measurements from 50 metaphase cells each from control, and Son-depleted (si1 and si4) samples showed increased spindle length in Son-depleted cells. (**C**,**K**) Son mRNA levels are significantly reduced in cells treated with *SON* siRNA duplexes compared with controls. Results are means ± s.d. DNA is stained with DAPI. Scale bar: 5 μm.

reorganization of nuclear speckle components following depletion of Son (Sharma et al., 2010). Interestingly, depletion of Son has exactly the opposite effect as SRSF1/SF2/ASF overexpression on BTM transcripts. Transient overexpression of SRSF1/SF2/ASF led to a higher inclusion of exon 6 than was observed in normal HeLa cells transfected with the β-tropomyosin minigene (Caceres et al., 1994). Also the subnuclear organization of noncoding RNA MALAT1 is altered in Son-depleted cells (Tripathi et al., 2010). Because MALAT1 regulates splicing factor distribution and activity, it will be interesting to know whether this nuclear-retained long noncoding RNA has any role in modulating Son-mediated alternative splicing changes.

Regulation of Son splicing impacts key cellular pathways

We and others have shown that depletion of Son causes decreased cell growth (Ahn et al., 2008; Sharma et al., 2010; Huen et al., 2010), mitotic arrest in metaphase (Sharma et al., 2010; Huen et al., 2010), and altered chromosome dynamics with spindle assembly checkpoint activation (Huen et al., 2010). It is possible that Son has as yet unidentified direct roles in mitotic structures. Because Son is required for proper splicing factor organization during interphase (Sharma et al., 2010), metaphase arrest in Son-depleted cells could result from the inability to organize splicing factors into mitotic interchromatin granule clusters that begin to assemble at metaphase (Ferriera et al., 1994). Our microarray data identified

Table 3. Genes showing altered splicing after depletion of Son

Symbol	Name	Fold change	*P* value	Exons	Splicing event
PITRM1	Metalloprotease 1	3.1	3.27E-04	E22-1	Alt-3′ and exon exclusion
SELENBP1	Selenium-binding protein 1	2.73	1.66E-04	E6-4	Exon region exclusion
LLGL1	Lethal(2) giant larvae protein homolog 1	2.73	2.33E-04	I17-1	Intron retention
ALDH3A1	Aldehyde dehydrogenase 3	2.58	1.60E-04	I6-1	Intron retention
SERINC2	Serine incorporator 2	2.55	4.36E-03	E4-2	Alt-5′ and cassette exon
DDX41	Probable ATP-dependent RNA helicase DDX41 (Dead box protein 41)	2.44	3.41E-04	E3-11	Exon-region exclusion and intron retention
VPS39	Vam6/Vps39-like protein	2.43	3.46E-04	I18-1	Intron retention
ALDH3A1	Aldehyde dehydrogenase 3	2.43	3.88E-06	I6-2	Intron retention
GMPPA	Mannose-1-phosphate guanyltransferase alpha	2.41	3.19E-03	E4-2	Exon-region exclusion
DDX41	Probable ATP dependent RNA helicase DDX41 (Dead box protein 41)	2.40	3.95E-04	E3-3	Exon-region exclusion
SNX11	Sorting nexin-11	–3.29	6.79E-06	E2-1	Alt promoter and cassette exon
PLCH2	Phosphoinositide phospholipase C	–2.97	1.35E-04	E25-11	Bleeding exon
PILRB	Activating receptor PILR-β	–2.69	6.44E-04	E10-3	Alt5 and cassette exon
C1orf2	Protein COTE1	–2.67	1.40E-06	E3-1	Cassette exon
CLCNKB	Chloride channel Kb	–2.61	6.07E-06	E11-1	Cassette exon
PDE4B	cAMP specific 3′,5′-cyclic phosphodiesterase 4B	–2.60	1.72E-03	E11-1	Alt-C-terminus
CCDC34	Coiled-coil domain-containing protein 34	–2.57	8.82E-06	E3-2	Bleeding exon
PHYHD1	Phytanoyl-CoA dioxygenase domain-containing protein 1	–2.54	6.25E-04	E8-1	Cassette exon
GPR175	Integral membrane protein GPR175	–2.50	3.57E-06	E9-1	Cassette exon
ADA	Adenosine deaminase	–1.80	1.09E-07	E9-1	Exon-region exclusion
HDAC6	Histone deacetylase 6	–1.17	8.50E-05	E27-2	Exon-region exclusion
HDAC6	Histone deacetylase 6	–1.04	1.97E-03	E28-1	Exon-region exclusion
SETD8	Histone-lysine N-methyltransferase	–1.05	4.17E-04	E2-2	Exon-region exclusion

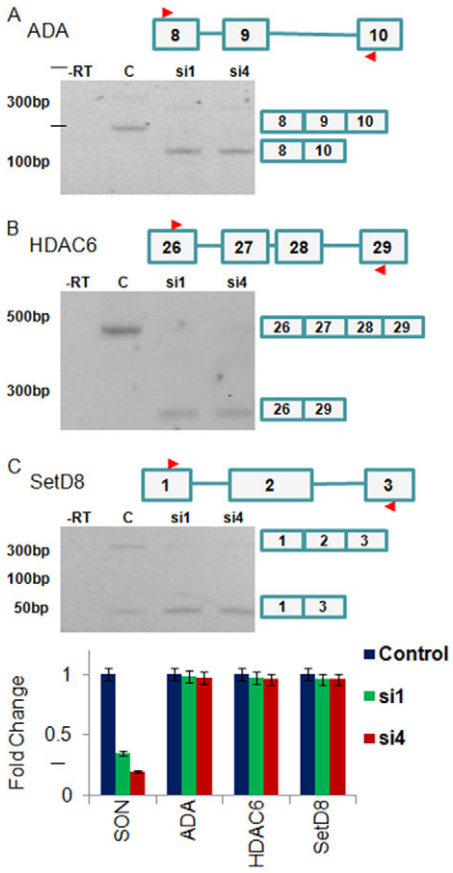

Fig. 9. Son depletion causes changes in splice-site selection of chromatin modifying enzymes. RNA samples used to perform the exon array were used to show a change in splice site selection in *ADA* (**A**), *HDAC6* (**B**) and *SETD8* (**C**) transcripts after Son depletion with two separate siRNAs (si1 and si4) targeting *SON* compared with control siRNA treatment (C). No transcripts were observed when reverse transcriptase was omitted from the reactions (-RT). The levels of *ADA*, *HDAC6* and *SETD8* transcripts were similar between Son-depleted samples and control samples (see graph at bottom). Results are means ± s.d.

many transcripts for cell cycle regulators (including mitotic regulators) that were affected by reduced expression of Son. According to our results, depletion of Son caused exon skipping in *HDAC6* transcripts. HDAC6 not only deacetylates histone tails to regulate gene expression (Narlikar et al., 2002), but it also deacetylates tubulin at Lys40 and thereby influences the stability of the microtubule network (Zhang et al., 2003; Zhao et al., 2010). Moreover, HDAC6 inhibitors induce degradation of both Aurora A and B kinases through a proteasome-mediated pathway and induce apoptosis following G2–M arrest (Cha et al., 2009). Other transcripts that are altered after knockdown of Son include *KATNB1*, which is involved in microtubule depolymerization (Yu et al., 2005) and *MAP2* (Itoh and Hotani, 2004). Our exon array results indicate that Son also regulates mRNA levels and splicing of cyclin-dependent kinases such as *CDK6* and *CDK2*, which promote transition between phases of the cell cycle (Ekholm and Reed, 2000). The effect of the impact of Son on mitotic progression was recently shown elsewhere, and also indicated that Son maintains microtubule organization through the cell cycle (Ahn et al., 2011). Our results also suggest that Son maintains proper microtubule organization, in particular during mitosis, when reduction of Son expression leads to increased interpolar distance and disorganized spindle microtubules. Observations in all of these studies are consistent with the idea that Son promotes proper cell cycle progression by maintaining proper transcription and splicing regulation for a subset of cell cycle regulator mRNAs.

Recently, Ahn and colleagues (Ahn et al., 2011) reported microarray analysis for Son-depleted cells that showed changes in constitutive splicing output, including some changes that were also detected in our study. The genes Ahn and co-workers identified showed negative expression changes in the overlapping categories of ‘DNA replication, recombination, and repair’ and ‘cell cycle’ were all confirmed by our data, and show changes in the same direction to approximately the same degree: *AKT1* (–1.9-fold), *CHFR* (–1.2-fold), *SMC1A* (–1.3-fold), *TYMS* (–1.5-fold) and *CDC45L* (–2.2-fold). Ahn and colleagues validated constitutive splicing defects for the genes *AKT1*, *KATNB1* and *TUBG2*, and we also detected changes in these genes. However, there are differences in the position within the transcripts where defects occur as well as in the variety of transcripts showing changes in mRNA levels. For example, our data set indicates changes in the tubulin isoforms *TUBA1A* (a ‘cell cycle’ hit), *TUBB6* and *TUBG2*, whereas they noted changes in *TUBG1* and *TUBA1B*. We did not see significant changes in splicing of *PCNT*, *AURKB*, *RAD23A*, *FANCG* or *AURKA*, which showed constitutive splicing defects in the report by Ahn and co-workers (Ahn et al., 2011). Moreover, *KATNB1* is one of the genes that indicated intron retention in both studies, although intron 9 was retained in our study, whereas intron 14 was retained in the other study. *AKT1* transcript was also altered in both studies, although intron 13 was retained in our study whereas intron 11 was altered in the other study. Differences observed for mitotic regulator mRNAs between the two microarray data sets are possibly due to the different timing of RNA harvest following Son depletion, which would probably change the ratio of cells undergoing mitotic arrest and/or cell death [48 hours as performed here versus 66 hours in the other report (Ahn et al., 2011)], as well as the different types of gene chips that were used, so caution should certainly be taken when making direct comparisons between data sets. Ultimately, the common trends observed in both studies such as the defects observed in cell cycle regulator transcripts will point toward the most relevant targets for follow-on studies.

Our study demonstrates alternative splicing defects in a group of chromatin modifier transcripts. We validated altered splice site selection on *ADA*, *HDAC6* and *SETD8* transcripts that have not been reported elsewhere. Our results show that Son maintains proper splicing of transcripts for *SETD8*, which regulates gene expression in a cell-cycle-dependent manner by mono-methylation of histone 4 at Lys20 (H4K20me1) to promote chromosome condensation (David, 2010). Son also maintains splicing of transcripts for *ADA*, which has been implicated in yeast to be a part of a histone acetyl transferase complex (HAT) (Grant et al., 1997). Additional genes that show significant changes after Son depletion include DNA methyltransferase 1 (*DNMT1*) and DNA methyltransferase 1-associated protein 1 (*DMAP1*), which are involved in DNA methylation (Rountree et al., 2000) or transcription repressors such as B-cell lymphoma 6 (*BCL6*) (Ahmad et al., 2003) and MYST histone acetyltransferase (monocytic leukemia) 4 (*MYST4*) (McGraw et al., 2007). Epigenetic modifications could therefore be altered in Son-depleted cells as a result of inappropriate protein expression for these modifiers. This is clear for all three of our validated

examples, because improper splicing would lead to deletion of the zinc-finger domain in HDAC and deletions in the coding regions of both *ADA* and *SETD8* transcripts.

Epigenetic modification could in turn affect splicing choices by Son, because splicing adaptor proteins read epigenetic signatures and regulate splice site choice (Kornblihtt et al., 2009; Luco et al., 2010; Luco et al., 2011). Son appeared among the top ten most significant hits for stem cell maintenance when it was identified as a pluripotency factor in human embryonic stem cells (Chia et al., 2010). Now that we have identified Son transcription and splicing targets genome wide, we can begin to tease apart the roles for Son as a crucial regulator for pathways such as cell growth, signaling or gene regulation, that might ultimately determine cell fate. It is interesting to consider that 'cellular memory' for pre-mRNA splicing might be programmed by epigenetic status, such that heritable chromatin marks could specify splice site selection in daughter cells. The discovery that Son is required for maintaining pluripotency of human embryonic stem cells places it as a key player for controlling such outcomes.

Materials and Methods

Cell culture

HeLa cells were maintained in DMEM supplemented with penicillin-streptomycin and 10% fetal bovine serum. HeLa cells stably expressing rat β-tropomyosin minigene were described previously (Prasanth et al., 2003).

RNA-FISH and immunofluorescence

RNA-FISH was performed as previously described (Prasanth et al., 2003; Sacco-Bubulya and Spector, 2002). β-tropomyosin minigene transcripts were detected by RNA-FISH with a 30-nucleotide probe conjugated with a single Texas Red molecule at the 5′ end (Sigma) designed to hybridize to exon 5. A 24-nucleotide splice junction probe conjugated to a single molecule of Cy5 at the 5′ end (Sigma) was designed to hybridize to reporter transcripts lacking exon 6. RNA-FISH was performed using stringent hybridization conditions that did not permit partial probe hybridization (e.g. cells were probed in parallel with equal mixture of two Cy5-labeled probes targeting the 12 nucleotides at the 3′ end of exon 5 or the 12 nucleotides at the 5′ end of exon 7 (Sacco-Bubulya and Spector, 2002). Immunofluorescence was performed as previously described (Sharma et al., 2010) following RNA-FISH, with the exception that 0.5 % bovine serum albumin in RNase-free phosphate-buffered saline (PBS) was used as a blocking agent to maintain RNase-free conditions. YFP–Son FL (Sharma et al., 2010) and other deletion constructs of Son were transected 48 hours prior before performing RNA-FISH using a calcium phosphate method, as described previously (Sharma et al., 2010). Exogenous YFP–Son was detected with monoclonal EGFP antibody (1:100; Clontech) following RNA-FISH. Anti-Son antibody (Sharma et al., 2010), monoclonal anti-SRSF1/SF2/ASF antibody AK103 (1:2500; provided by Adrian Krainer, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), human anti-fibrillarin antibody (1:20, Sigma), PML antibody 5E10 (1:20) (Stuurman et al., 1992) and monoclonal anti-U1-70K 2.73 (1:100; provided by Sally Hoch, Agouron Institute, La Jolla, CA) were diluted in 0.5% bovine serum albumin in RNase-free PBS. Fluorescently conjugated secondary antibodies (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) were diluted in 0.5% bovine serum albumin in RNase-free PBS. Image *z*-stacks through cell nuclei were collected on a DeltaVision RT imaging system (Applied Precision) equipped with a 60× Plan-Apo objective (1.4 NA). Figures contain projections of raw RNA-FISH image data. Individual BTM loci were frequently observed in close proximity to nucleoli. Depending upon nucleolar orientation, the locus occasionally appears to be situated on the interior of the nucleolus in *z*-stack projections; however, in all cases it actually lies in the upper or lower side of the nucleolus. Microtubules were labeled using anti-α-tubulin (Sigma) and displayed as projections of deconvolved *z*-stacks. Interpolar distances were measured in three dimensions (linear distance through the *z*-stack) using SoftWorx image analysis software (Applied Precision).

RNA interference and RNA sample preparation

RNAi-mediated knockdown of Son was performed as previously described (Sharma et al., 2010). Briefly, Oligofectamine (Invitrogen) was used according to manufacturer instructions for transfection of HeLa or HeLa BTM cells with 60 pmol siRNA duplexes targeting *SON* (siGENOME1 and siGENOME4; Dharmacon) or 60 pmol control siRNA duplexes targeting luciferase in antibiotic-free medium. Cells were harvested 48 hours after siRNA transfection. RNA extraction was performed according to RNAeasy (Qiagen) kit protocols, and DNA was removed using Turbo DNA-Free kit (Ambion). Because our anti-Son antibodies do not detect Son by western blot, we routinely scored only the siRNA-treated cells in which the immunofluorescence signal for Son was significantly reduced compared with controls. In addition, for every fluorescence experiment, samples were prepared in parallel for RNA extraction and qRT-PCR to verify Son depletion.

Exon array

Five replicates of the Human Exon Array 1.0 ST were performed using RNA samples isolated from five independent RNAi experiments (Son-siRNA4- or control-siRNA-treated cells). Each RNA sample was subjected to DNase treatment as described above. RNA quality was determined by microfluidic analysis, using the Agilent 2100 Bioanalyzer, a RNA 6000 Nano Kit (PN 5067-151,1Agilent; CA) to ascertain that the RNA was of sufficient quality for further analysis. RNA integrity numbers were all greater than 9.0. A Whole Transcript (WT) Expression Array kit (Ambion) was used to generate biotinylated sense strand DNA targets from the isolated RNA as described in the kit protocol. DNA quality and quantity was analyzed by nanodrop spectrophotometry (Thermo Scientific) and subjected to fragmentation and labeling using the Affymetrix GeneChip WT terminal labeling kit according to kit protocols. Each sample was hybridized and subjected to an automated washing, staining and scanning on the Fluidics Station 450 (Affymetrix) following fluidics protocol FS450-0001.

Array results were analyzed for differential gene expression and alternative splicing in control versus Son-depleted samples. AltAnalyze (Emig et al., 2010) assessed genome wide changes in transcription as well as alternative splice events. Constitutive exons were used to detect the changes in transcription, filtering out probe sets with a Detection Above Background (DABG) *P* value of greater than 0.05 and probe sets with a non-log expression less than 70. The splicing index algorithm (the log ratio of the exon intensities between samples) and the MIDAS algorithm (uses the ANOVA model on the splicing index) was used to predict alternative splicing (Gardina et al., 2006; Srinivasan et al., 2005). Benjamini–Hochberg correction was applied to eliminate false positives (Benjamini and Hochberg, 1995). The primary data have been deposited in the NCBI GEO database under the accession number GSE28672.

Quantitative RT-PCR

Quantitative RT-PCR was performed using BR 1-Step Sybr Green qRT-PCR kit (Quanta) and 100 ng of total RNA. GAPDH primers 5′-ATGTTCGTC-ATGGGTGTGAA-3′ and 5′-GGTGCTAAGCAGTTGGTGGT-3′ were used as an internal control for all qRT-PCR reactions. Son depletion was validated using primers 5′-GTACCCTGAGCCAAGCACAT-3′ and 5′-GGCTGCTCTGGCAAT-CTTAG-3′. Primer sets used to detect β-tropomyosin transcript levels were as follows: exon5–intron5 (5′-AGCTGGTGATCCTGGAAGG-3′ and 5′-ACCCG-GGTATCCCTACCTC-3′); intron-5–exon-6 (5′-AGGCCACAGGAATAGGCT-TT-3′ and 5′-CCATGGTTCGAAGCTCCTC-3′); exon-6–intron-6 (5′-CAG-GCTCTCAAGTCGCTGAT-3′ and 5′-AGGTGCCATTGCCTAGAAAG-3′); exon-5–exon-6 (5′-CTGAAGAGAGAGCCGAGGTG-3′ and 5′-GACTTGAGAGCC-TGGTCCAT-3′); exon-5–exon-7 (5′-CTGAAGAGAGAGCCGAGGTG-3′ and 5′-CCTCCTCTAGGTCCCCACAT-3′); exon-5 (5′-CCAGGAAGCTGGTGATCCT-3′ and 5′-AGCCACCTCGGCTCTCTC-3′); and intron-5 (5′-TGGAACGAAT-GGGATGATAGA-3′ and 5′-AGCCTATTCCTGTGGCCTCT-3′). qRT-PCR products were analyzed by electrophoresis on 12% native polyacrylamide gels. Primers used for validating exon array results are shown in supplementary material Table S1.

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